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CYTOLOGIC EFFECTS OF AIR FORCE CHEMICALS

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

A. A. Thomas

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Director
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MICRONUCLEUS	JP-5	

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EXPERIMENTAL ANIMALS HAVE BEEN EXPOSED TO MATERIALS OF INTEREST TO THE AIR FORCE AND THEN EXAMINED FOR EVIDENCE OF GENETIC DAMAGE. MATERIALS STUDIED INCLUDED MONOMETHYLHYDRAZINE, JP-5, AND DIESEL FUEL MARINE. EXPOSURE TO MODERATE LEVELS OF THESE MATERIALS BY INHALATION DID NOT PRODUCE DETECTABLE CHROMOSOMAL ABNORMALITIES. VARIOUS ADDITIONAL TESTS FOR GENETIC DAMAGE ARE BEING EVALUATED WITH THE VIEW TO DEVELOPING METHODS OF DETECTING POSSIBLE GENETIC HAZARDS TO HUMANS.

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PREFACE

This is the second annual report of the Cytology, Cell Biology and Cytogenetics Section of the Toxic Hazards Research Program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under contract AF33615-76-C-5005. This report describes the accomplishments from July, 1977 through June, 1978. During this period, T.T. Crocker, M.D. served as the principal investigator, R.E. Rasmussen, Ph.D. was the coordinator for the subprogram and operated the laboratory facilities at UCI, and R.D. Benz, Ph.D. and P.A. Beltz operated the laboratory facilities at the Toxic Hazards Research Unit (THRU) in Dayton, Ohio. K.C. Back, Ph.D., Chief of the Toxicology Branch was the technical monitor for the Aerospace Medical Research Laboratory.

This subprogram continues to be concerned with the detection and study of toxic effects of compounds of interest to the Air Force and other agencies as these effects may be manifested at the cellular or subcellular level in exposed animals, including humans. Among its goals are the study of chromosome abnormalities, detection of mutagenic or carcinogenic events, activation or inactivation of detoxifying enzymes, and effects on DNA synthesis and repair.

Activities at the THRU have been concerned with the development and application of cytogenetic test procedures to animals exposed by inhalation and also to peripheral blood exposed in vitro. Three tests, the micronucleus test, the sister-chromatid exchange test, and the chromosome aberration test, are being used to monitor possible genetic damage.

Complementary studies are underway at UCI in which other in vivo and in vitro tests are being evaluated to determine which may be of use in the detection of genetic damage produced in experimental animals as the result of exposure to materials of military and industrial importance.

SUMMARY

At the THRU laboratories at WPAFB three tests for cytogenetic damage have been developed and applied to experimental animals (dogs and rats) exposed by inhalation to materials of interest. The tests were all designed to detect chromosome damage which could be observed microscopically, and included sister-chromatid exchange, micronucleus assay, and chromosome aberration analysis. A proven clastogenic agent, ethylmethane sulfonate, was used in vitro as a positive control to demonstrate the sensitivity of the assays. At the concentrations used, none of the test materials produced detectable chromosome damage above the background level.

Preliminary tests using small peripheral blood samples from human volunteers have established the feasibility of routine monitoring of persons exposed occupationally to possibly cytogenetically-hazardous materials.

In complementary studies at UCI, biochemical studies have been done in order to estimate whether genetic damage which might be expressed in alteration of specific function may have been produced in animals exposed to the test materials. The functions examined were DNA replication and repair, and the activities of enzymes concerned with drug and carcinogen metabolism. The effect of inhalation exposure to MMH and JP-5 on these functions was minimal but some effects were noted.

Preliminary results suggest that inhalation of MMH stimulates cellular replication in tracheal and urinary bladder epithelium. In animals injected with MMH, there may be a transient stimulation of drug metabolizing enzymes in the liver. In mice and rats exposed by inhalation to JP-5, no differences in any function have been found between treated and controls. However, there was some indication of induction of drug-metabolizing enzymes in mouse liver when JP-5 was injected i.p. These effects are presently under continuing study.

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R.D. Benz, Ph.D.

INTRODUCTION

Damage to the genetic material of a cell, the DNA molecules and their associated proteins, can be divided into two kinds. One kind is point mutations. These are small deletions of one (or a few) bases in the DNA molecule in a chromosome, or chemical changes in one (or a few) base(s) causing it (them) to pair differently when new DNA is synthesized. These result in a change in the DNA sequence which results in different information being presented for use in the cell. The other kind of damage is chromosome breaks. These are gross changes in the structure of one or more chromosomes such as losses of pieces or exchanges of pieces. The difference between these two general kinds of damage is one of scale, since very large deletions in the DNA molecule indeed are losses of small chromosome pieces.

As yet we have not studied point mutation in this subprogram although we hope to expand into this area in the next year. We have studied chromosome breakage, however, in three assay systems.

Four things can happen if a chromosome is broken: 1) The piece may be perfectly repaired at its original location. This event can be detected by monitoring DNA synthesis which occurs as part of the repair process (an unscheduled DNA synthesis assay); 2) The piece may be repaired, but during the repair process it is exchanged with the analagous piece on the other arm of the chromosome (the sister-chromatid). This event can be detected by monitoring the exchange of parts of two sister-chromatids which have been differentially stained (a sister-chromatid exchange assay); 3) The piece may be repaired, but not at its original position, e.g. the piece from one chromosome becomes attached to a completely different chromosome. This can be detected by closely examining metaphase chromosomes for unusual morphologies (a chromosome aberration assay); 4) The piece may remain unrepaired. This can be detected in mitotic cells with a chromosome aberration assay, but the broken piece can also be detected as a small, separate nucleus in interphase cells (a micronucleus assay).

SISTER-CHROMATID EXCHANGES AND CHROMOSOME ABERRATIONS

When a broken chromosome piece is exchanged for the identical piece on the other arm of the same chromosome during repair, a sister-chromatid exchange (SCE) has occurred. Recently, a method has been developed (Latt, 1973; Perry and Wolf, 1974) by which one chromosome arm of a given chromosome can be stained very lightly while the other arm is stained darkly (Figure 1). This differential staining is obtained by growing dividing cells in a medium containing bromodeoxyuridine (BrdU). The cells use the BrdU in place of thymidine (one of the four normal sub-units of DNA) in synthesizing new DNA. After two replicating periods, there are bromine atoms in both halves of the DNA double helix in one arm of each chromosome in a cell, but only in one of the two DNA halves in the other chromosome arm. When the cells are

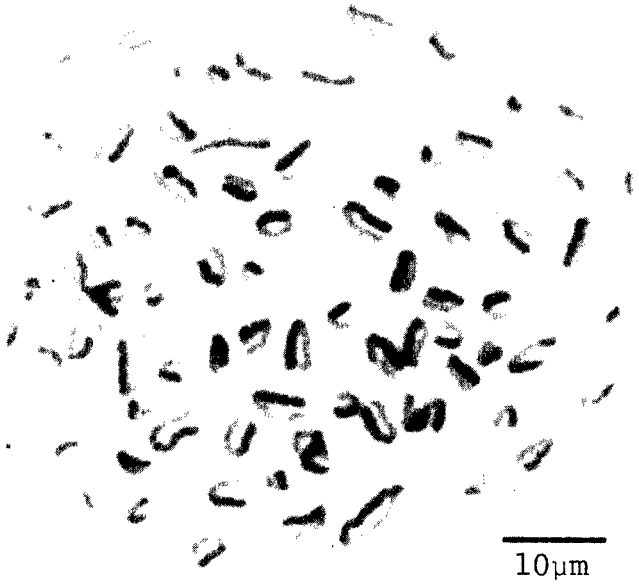

Figure 1. Canine peripheral lymphocyte metaphase chromosomes stained to reveal sister-chromatid exchanges.

stained with a particular DNA stain* that is proportionately quenched by the presence of bromine and then counterstained with Giemsa blood stain, one can see chromosomes with one light arm and one dark arm when observed under a light microscope. If one or more parts of chromosome arms have been induced to exchange with their sister-chromatids while growing under these conditions, however, a harlequin effect results. A darkly stained chromosome arm now has a light end and vice versa. These exchanges are easily seen under the microscope. At the same time, while checking for these exchanges, chromosome aberrations can be scored.

In the past year, we have attempted to develop a procedure for detecting induced sister-chromatid exchanges and chromosome aberrations in peripheral lymphocytes of dogs exposed to various substances and their controls. We have encountered considerable difficulty, however, because canine lymphocytes seem to have an unusually rigid nuclear area making it quite difficult to obtain satisfactorily spread chromosomes. Since dogs have 78 chromosomes, when the chromosomes are not well-spread out and separated, it is impossible to reliably detect any details along their lengths. It becomes like trying to pick out all of one strand in a plate full of spaghetti. After trying a large number of combinations of techniques, we have developed a procedure that results in producing scoreable canine mitotic chromosomes that are properly stained for SCE detection in about one-third of attempts. The procedure was adapted from one developed by Chaganti, Schonberg and German (1974) for use with human lymphocytes.

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* The stain is Hoechst 33258 or, chemically, 2-[2-(4-hydroxyphenol)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazole.

Methods

One-tenth milliliter of freshly-drawn whole blood is cultured in 3 ml McCoy's 5A cell medium containing 20% fetal calf serum for extra nutrients, 50 units/ml penicillin and 500 µg/ml streptomycin to retard bacterial contamination, 7 units/ml heparin to prevent clotting, 10 µM BrdU, and 0.1 ml rehydrated GIBCO phytohemagglutinin-M to stimulate lymphocyte division, at 37° C, 95% relative humidity, and 5% CO_2 atmosphere for 49 hr. After this time, when many cells are about to enter mitosis for the second time, colchicine is added to make the final concentration 10^{-6} M colchicine, and the incubation is continued for 4 hr additional while the mitotic cells accumulate. At this point, the culture tubes are centrifuged at 280 g for 5 min and the cell medium is removed from the cell pellet. Five milliliters of 75 mM KCl (at pH 4.5, 37° C) are added slowly with gentle mixing to the cell pellet and then left to stand for 8 min. The red cells swell and explode under this treatment and are removed from the population. The white cells swell, but do not break, and the chromosomes within spread. The cells are then centrifuged and the supernatant removed. Seven milliliters of a 3:1 mixture of ethanol and acetic acid ("fix") are added to the cell pellet with prompt, thorough mixing and then left to stand 10 min. This treatment preserves the chromosome morphology. This step is repeated after centrifugations and removals of the supernatant two more times using 5 and then 2 ml of fix. After the last centrifugation, the cells are dropped from a 1 m height (to smash the spherical cells flat) onto cold, alcohol-washed slides that have been dipped in water. After the slides have been allowed to dry, they are stained for 12 min with 50 µg/ml Hoechst 33258 under bright cool white fluorescent illumination and then are covered and sealed with rubber cement and exposed to intense illumination for an additional 3 hr. After this time, the slides are unsealed and are stained with 3% Giemsa blood stain for 10 min, rinsed, and then permanently sealed under coverslips.

Experimental Results and Conclusions

To date, successfully prepared canine chromosome preparations have been obtained for statistically useful results on only two occasions. The first of these was while testing blood samples from dogs that had been exposed by inhalation to diesel fuel marine (DFM) vapor continuously for thirteen weeks (Table 1). No significant induction of SCEs above background by DFM was observed and no chromosome aberrations were seen. The second successful results were obtained with a pre-exposure sampling from dogs that were to be exposed continuously to decahydronaphthalene (decalin). These data have not been completely analyzed at this time.

The sister-chromatid exchange assay is by far the most sensitive assay of chromosome damage now developed in mammalian systems (Perry and Evans, 1975). It is as much as one thousand-fold more sensitive than assays where broken chromosome pieces (chromosome aberrations or micronuclei) are scored. It is therefore worthwhile to attempt to develop this assay even though it requires obtaining well-spread mitotic chromosomes and involves tedious scoring routines. Because of the very unreliable results thus far obtained using dog lymphocytes, less effort will be expended with this assay and species. Future efforts will be expanded to develop techniques to use this assay with other species.

TABLE I.

INDUCTION OF SISTER-CHROMATID EXCHANGES BY DIESEL FUEL MARINE

Animal Identification Number	Exposure Level (mg/liter)	Total Number of Metaphases Examined	Total Number of Chromosomes Examined (1)	Total Number of Exchanges Found	Number of Exchanges per Chromosome	Normalized Number of Exchanges per Cell (2)
X48	0.00	56	1253	59	0.0471	3.67
X88	0.00	53	1710	90	0.0573	4.47
X52	0.05	12	317	14	0.0442	3.45
Y10	0.05	2	68	1	0.0147	1.15
X14	0.30	20	417	25	0.0600	4.68

(1) Fewer than 78 chromosomes (the total number of chromosomes in a dog cell) were scorable in most metaphases.

(2) Number of exchanges per chromosome x 78.

MICRONUCLEUS TEST

It is possible to stop dividing mammalian cells in metaphase with the drug colchicine. After preparation for microscopic examination, mitotic chromosomes of cells can be examined and breaks observed directly. This procedure, however, is tedious since it is difficult to prepare cells with metaphase chromosomes that are well-spread out on the microscope slide. A technique has been developed, however, that overcomes this problem (Heddle, 1973; Countryman and Heddle, 1976; Heddle, Benz and Countryman, 1977). Instead of arresting the cells in metaphase, they are allowed to continue into the next interphase. If a broken chromosome piece is present in metaphase, quite often this piece will not be drawn into the nucleus at the next interphase since there is no spindle attached. Instead, this piece will form its own "micronucleus." Thus, to assay unrepaired chromosome breaks, interphase cells can be stained and scored for the presence of micronuclei.

In the past year we have adapted a very reliable procedure for detecting micronuclei (Countryman and Heddle,1977) in peripheral lymphocytes taken from dogs exposed to various chemicals and their controls (Figure 2).

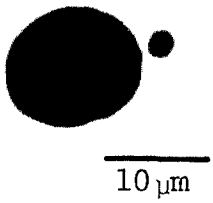

Figure 2. Canine lymphocyte nucleus with associated micronucleus.

Methods

Two-hundreths milliliter freshly-drawn whole blood is cultured in 1 ml McCoy's 5A cell medium containing 20% fetal calf serum, 50 units/ml penicillin, 500 μg/ml streptomycin, 12 units/ml heparin and 0.025 ml rehydrated GIBCO phytohemagglutinin-M at 37° C, 95% relative humidity, and 5% CO_2 atmosphere for 75 hr. After this time, the culture tubes are centrifuged at 12,000 g for 15 sec and the cell medium is removed from the cell pellet. One milliliter of 16 mM trisodium citrate (37° C) is added to the cell pellet and left to stand for 12 min. The cells are then centrifuged and the supernatant removed. One milliliter of a 3:1 mixture of ethanol and acetic acid ("fix") is added to the cell pellet with thorough mixing and then left to stand for 10 min. The cells are then centrifuged and most of the supernatant is removed. The cell pellet is resuspended vigorously in the remaining supernatant and then dropped from a 1 m height onto alcohol-washed slides that have been dipped in water. After the slides have been allowed to dry, they are stained with 3% Giemsa blood stain for 10 min, rinsed, and then permanently sealed under coverslips. The slides are then examined under 400x magnification for the presence of nuclei with associated micronuclei.

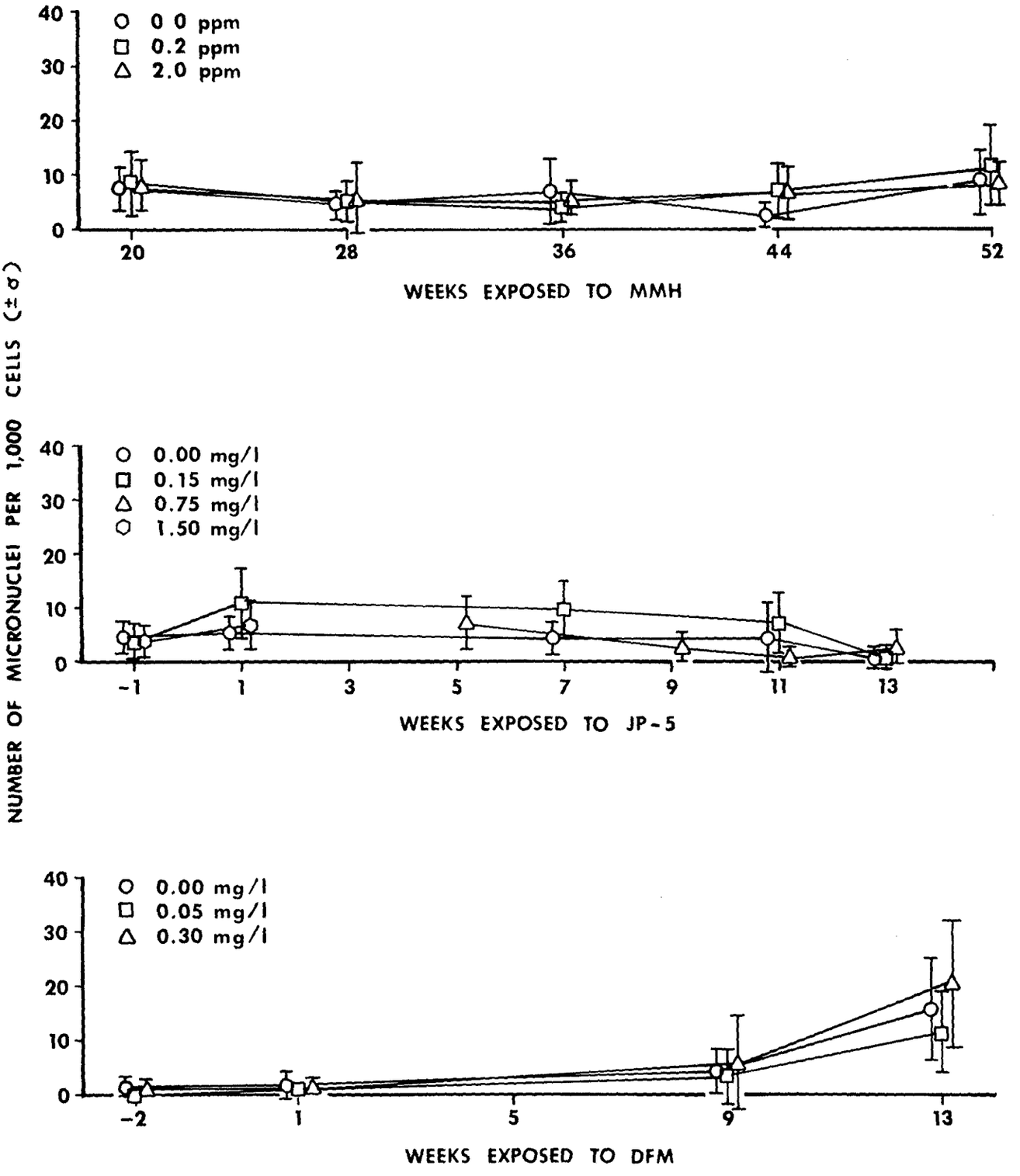

Figure 3. Induction of micronuclei by monomethylhydrazine. Figure 4. Induction of micronuclei by JP-5. Figure 5. Induction of micronuclei by diesel fuel marine.

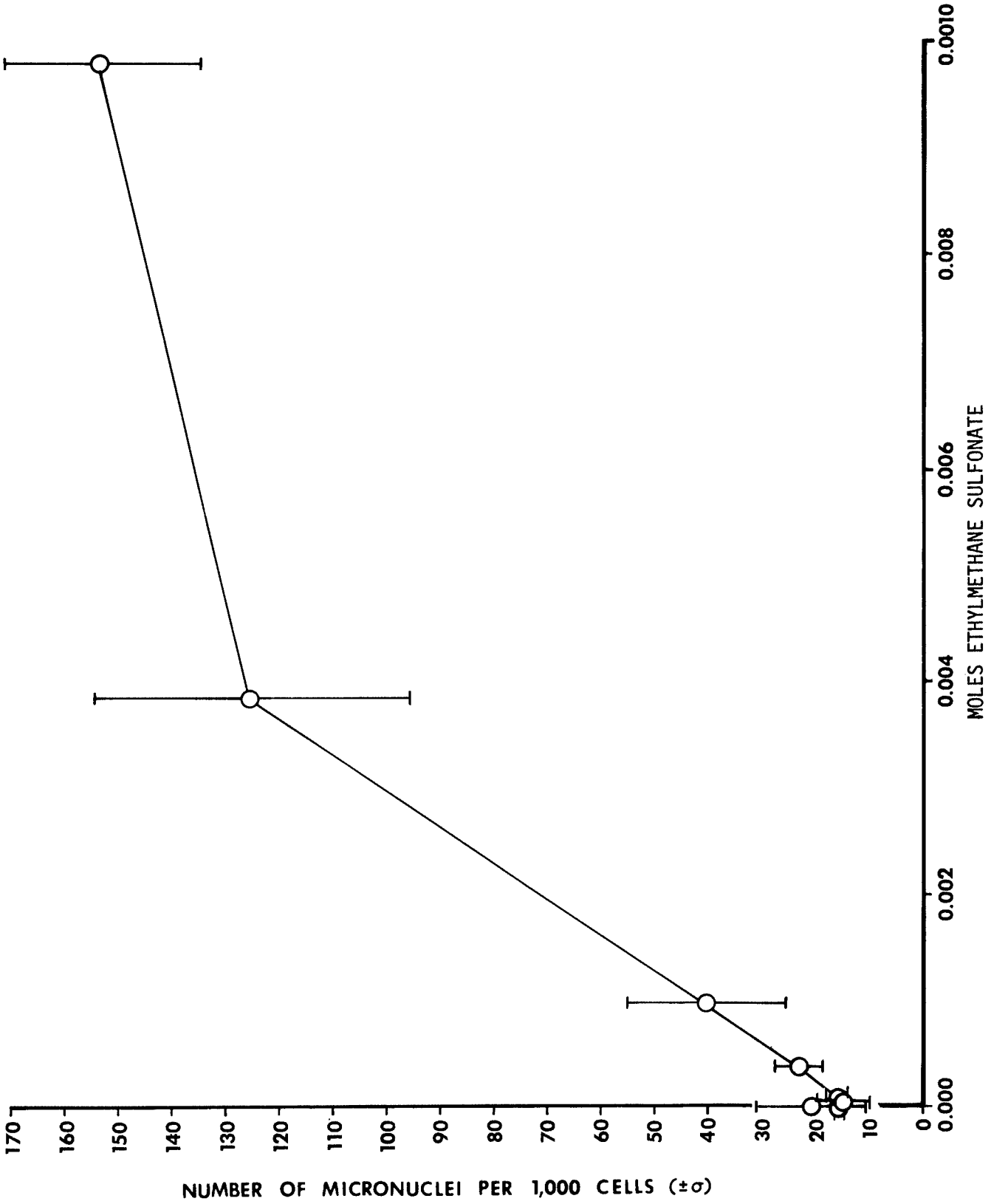

Figure 6. Induction of micronuclei by ethylmethane sulfonate.

Experimental Results and Conclusions

We have routinely used this procedure in assays for gross chromosome damage induced in lymphocytes of dogs exposed in vivo by inhalation for various lengths of time to monomethylhydrazine (Figure 3), JP-5 (Figure 4), diesel fuel marine (Figure 5), and decalin (data incomplete at this time). No significant induction of chromosome breaks was observed with these chemicals at any sampling time.

We have also done in vitro exposures of dog blood to known mutagenic chemicals (Figure 6; other data incomplete at this time) and some hydrazine compounds (data incomplete). A dose response was found for canine lymphocytes exposed in vitro to ethylmethane sulfonate that was essentially identical to that reported for human lymphocytes (Heddle, Lue, Saunders and Benz, in press).

Our next step with the micronucleus assay is to complete the in vitro testing of the chemicals (e.g. the rest of the hydrazines) never tested in this way, and if warranted by positive results in these tests, to test blood from animals acutely exposed to these compounds. Once this is complete, we will assess the sensitivity of this entire assay system and also compare the results to long-term results of general toxicologic findings with animals exposed to the same chemicals. We will then decide if it would be worthwhile to use the micronucleus assay in routine testing of untested chemicals.

CURRENT DEVELOPMENTS

Assays of Cytogenetic Damage Using Rats

Some toxic chemicals may adversely affect one species of animals but not others at a given dose level. It is therefore desirable to test several species with each chemical of interest in the hope that the effects of the chemical on humans, the ultimate interest, will be similar to at least one of the species tested. We have recently begun to extend our cytogenetic experiments to tests using rats.

At this time, we are only completing development of the basic techniques to produce good, scoreable rat cells and chromosomes, and have not yet tested any chemicals.

We are using three approaches with rats. One is to test peripheral lymphocytes in vitro. For this a rat is sacrificed and whole blood is obtained for treatment and culturing. Culture conditions are the same as for dogs, described for the SCE assay and the micronucleus assay, except that incubation times are different, though not finally established at this time. We have found that it is quite easy to obtain well-spread chromosomes using rat blood (Figure 7). The problem we have encountered is that it is not easy to stimulate rat lymphocytes to divide, but this problem has recently been solved by using a special mitogen, leucoagglutinin, instead of the usual phytohemagglutinin.

Another approach is to obtain very small peripheral blood samples from rats by drawing from an exposed subclavian vein.* In this

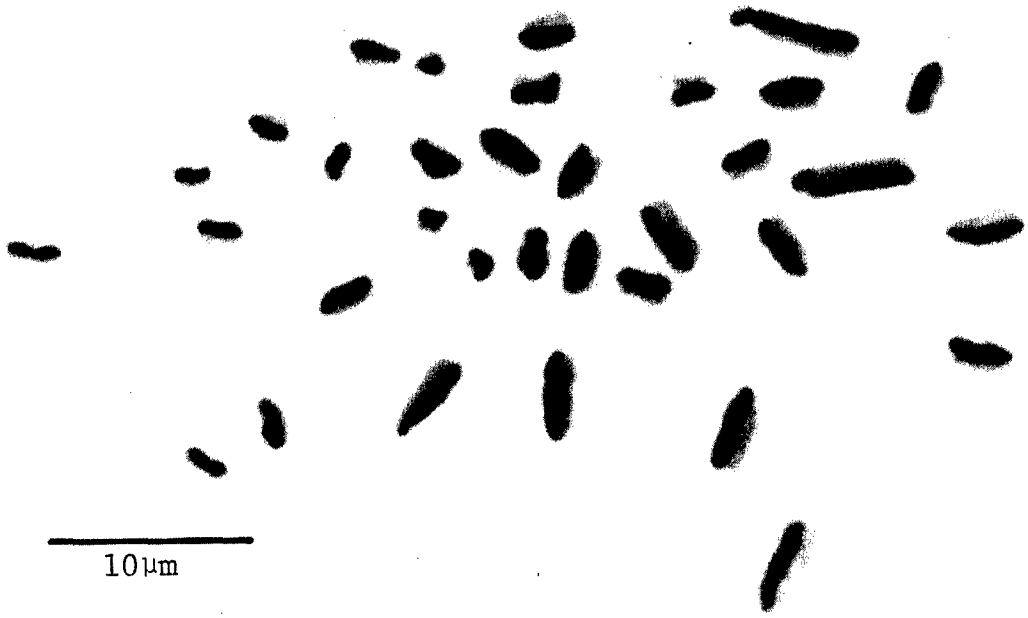

Figure 7. Rat peripheral lymphocyte metaphase chromosomes stained to reveal sister-chromatid exchanges

case the rats are not sacrificed and serial samples can be obtained from the rats while continuous exposures to a chemical occur. Development of this technique is continuing.

The third approach is to obtain bone marrow samples from rats by inserting a spinal needle past the kneecap of the rat into the marrow of the femur (Nemenzo, Pasi and Hine, 1975). We have found that this technique requires a delicate touch to get the needle into the right place, but when developed, serial samples of primary, dividing cells from continuously exposed animals will be obtained and used.

Cytogenetic Analysis of Rhesus Monkeys

We received an unexpected opportunity to prepare slides of peripheral lymphocytes of rhesus monkey blood for cytogenetic analysis. We obtained small whole blood samples from one female, one male, and one monkey of unknown sex (by physical examination). Using metaphase chromosome preparations from the known female and male monkeys as controls, we determined that the sex of the unknown monkey was female. This was in agreement with observations made in exploratory surgery done on the monkey by others.**

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* Nemenzo, J.H., personal communication. ** Hubbard, G., personal communication.

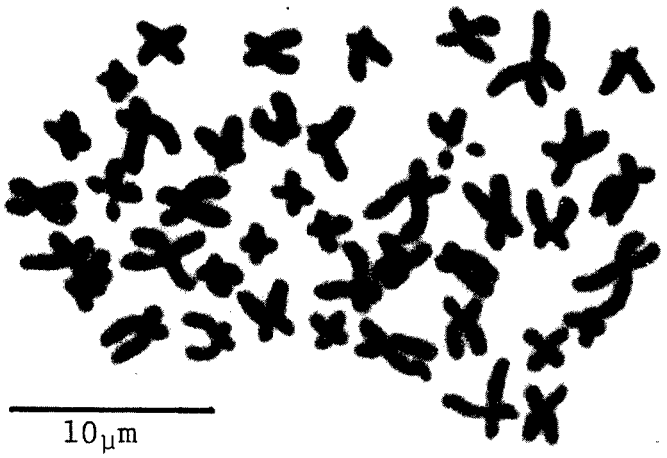

Figure 8. Rhesus monkey peripheral lymphocyte metaphase chromosomes.

In doing the preparations, we have established procedures for obtaining well-spread metaphase monkey chromosomes, should there be a requirement to use rhesus monkeys in cytogenetic testing assays in the future (Figure 8).

Monitoring Humans for Cytogenetic Damage

The ultimate goal in most toxicologic testing is to determine how the compounds will affect humans. We obtained small blood samples from eight persons having various duties at the THRU involving both exposure and non-exposure areas. We prepared the samples for cytogenetic analysis using well-established procedures for the SCE assay and the micronucleus assay with human cells. Scoring has been completed only for five of the eight persons in the SCE assay at this time (the micronucleus assay is complete), but all results so far have not differed from published background values (Chaganti, Schonberg and German, 1974) (Figure 9).

We are interested in the possibility of receiving blood samples from persons exposed chronically or acutely to suspect compounds for testing in the future and consider this current study as a good pilot run for such an endeavor.

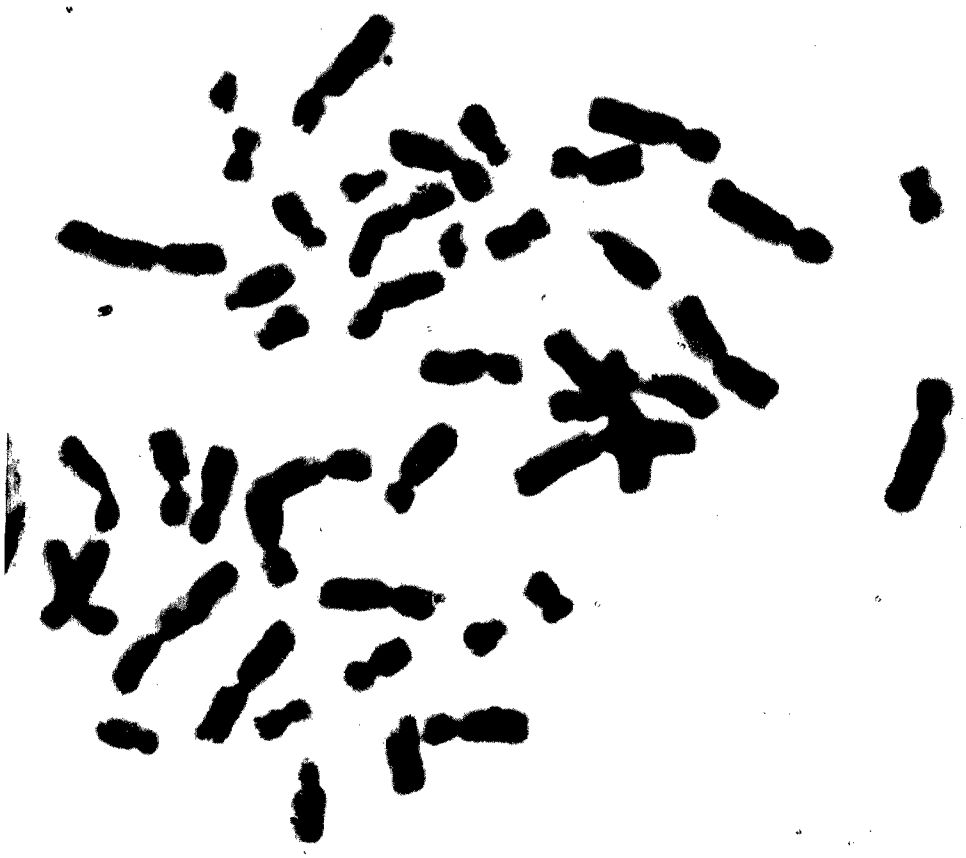

Figure 9. Human peripheral lymphocyte metaphase chromosomes stained to reveal sister-chromatid exchanges.

EXPERIMENTAL STUDIES AT UCI

R.E. Rasmussen, Ph.D.

STUDIES WITH MONOMETHYLHYDRAZINE (MMH) ADMINISTERED BY INHALATION

Methods

Inhalation Exposure. The exposure was performed at the THRU. Fifty male C57Bl/6J mice were exposed for 25 working days to 2 ppm of MMH. Upon completion of the exposure the mice were shipped by air to UCI for testing.

DNA Replication and Repair. These functions were measured in short-term organ culture. The mice were killed with an overdose of sodium pentobarbital, the lungs removed, and washed in cold buffered saline containing 1% fetal bovine serum. The tissues were then minced to fragments of approximately 1 mm^3, and 20 mg samples of the mince were incubated in buffered saline which contained ^{3}H-thymidine (5 µCi/ml, 60 Ci/mmole), hydroxyurea (10^{-2}M) to suppress replicative DNA synthesis, and with or without 4-nitroquinoline-1-oxide (4NQO, a DNA-damaging agent and lung carcinogen) to stimulate DNA repair. After incubation for 2-4 hr, the incorporation of label into the DNA was determined by a chemical extraction method which allows the measurement of the specific radioactivity of the DNA. The increased incorporation into the DNA of the tissue treated with 4NQO is taken as a measure of the DNA repair activity in that tissue during the incubation period, and is expressed as ^{3}H-DPM per µgm of DNA.

H-Thymidine Labelling Index. This was determined by injecting the mice i.p. with ^{3}H-thymidine (^{3}H-TdR, 100 µCi/animal, 60 Ci/mmole), killing the animals 1 hr later, and fixing and sectioning selected tissues for autoradiography.

Carcinogen Metabolizing Activity of liver and lung enzymes was measured in microsomal preparations from treated and control animals. The conversion of ^{3}H-benzo(a)pyrene to polar metabolites was measured using thin-layer chromatography. In order to test for the induction of carcinogen-metabolizing enzymes (also called aryl hydrocarbon hydroxylase, AHH), MMH-treated and control mice were injected with 0.2 ml corn oil containing 2 mg of 3-methylcholanthrene (3-MC) or with corn oil only. After 48 hr, liver and lung were removed and microsomes prepared by centrifugal fractionation of tissue homogenates. The ability of the microsomes to metabolize ^{3}H-benzo(a)pyrene to polar metabolites was measured. The incubation mixture contained 100 µCi ^{3}H-BP (1 µg), 1 mg microsomal protein in a total volume of 1 ml of tris-MG buffer, pH 7.4. After 5 min of aerobic incubation at 37° C, the reaction was stopped by rapid chilling in ice and the addition of 3 ml of ethyl acetate. The mixture was shaken vigorously, centrifuged to separate the phases, and the organic phase containing ^{3}H-BP and its metabolite removed. The organic extract was reduced in volume under N_2 and the residue applied to a thin-layer silica gel plate. The TLC plates were developed sequentially in the same direction with benzene and benzene:ethanol (19:1) to separate unchanged ^{3}H-BP from its metabolites.

Experimental Results and Conclusions

DNA Replication and Repair. Table 2 summarizes the results of this test. No difference was found in the DNA repair capacity of lung tissue between treated and control animals when the tissue was challenged in vitro with the lung carcinogen 4-nitroquinoline-1-oxide (4NQO). Some depression of DNA replication is indicated, but this was not supported by the ^{3}H-thymidine labelling index result. The conclusion is that the exposure to MMH had no significant effect on the ability of lung tissue to respond to the DNA-damaging agent used in these tests.

These results should not be taken as conclusive, because we have found in other studies (unpublished) that chronic exposure to cigarette smoke reduces the ability of mouse lung tissue to repair DNA damaged by 4NQO.

Table 2.

DNA repair and replication in lung tissue of C57Bl/6 mice exposed to MMH at the THRU. The values are ^{3}H-DPM per µgm of DNA, and are the average of triplicate samples.

	DNA Repair	DNA Replication
MMH-Treated	258	1950
Control	272	3130

^{3}H-Thymidine Labelling Index. Tissues examined included trachea, lung, liver, proximal small intestine. large intestine, kidney, spleen, and urinary bladder. No gross differences in morphology were seen between tissues from treated and control groups. Differences in labelling index were seen in the tracheal epithelium and the bladder epithelium, but in no other tissues (Table 3). The labelling index in lung was determined by counting cells in the alveolar regions of the lung; bronchial epithelium was excluded. The lung index did not correlate with the replicative synthesis measurements indicated in Table 2. Because of the limited number of animals examined in this study, the experiments should be repeated. These preliminary findings of increased cell proliferation in the bladder and trachea may be of special importance as early indicators of toxicity.

Carcinogen Metabolizing Activity. The results are shown in Table 4 and are expressed as the percentage of ^{3}H-BP converted to polar metabolites in 5 min. The values are based on duplicate samples.

The results indicate that the exposure to MMH did not impair the response of mice to 3-MC, or result in decreased basal levels of AHH. It was not possible to determine whether MMH was an inducer of AHH under the exposure conditions because the half-life of induced AHH enzymes in the absence of an inducer is much shorter than the transit time from the THRU to UCI. Further experiments were done to examine whether MMH may have transient effects on liver and lung enzymes that would not have been detected in these studies.

TABLE 3.

^{3}H-thymidine labelling index in selected tissues of C57Bl/6 mice exposed to MMH by inhalation at the THRU.

Tissue	Labelled/Unlabelled Cells
Trachea-MMH	25/1000
Trachea-Control	7/1000
Lung-MMH	2.6/1000
Lung-Control	2.9/1000
Bladder-MMH	124,52*
Bladder-Control	32,18*

TABLE 4.

Metabolism of ^{3}H-Benzo(a)pyrene by microsomes from C57Bl/6 mouse liver and lung tissue. Mice were exposed to MMH by inhalation as described. The values are percentage of input of ^{3}H-Benzo(a)pyrene converted to polar metabolites in 5 min.

	Liver Microsomes		Lung Microsomes	
	Uninduced	3-MC-Induced	Uninduced	3-MC-Induced
Control	0.167	0.61	0.019	0.019
MMH-treated	0.152	0.64	0.014	0.024

EFFECTS OF MMH GIVEN SYSTEMICALLY ON DNA REPLICATION IN THE HAMSTER LUNG

Methods

Adult golden Syrian hamsters were treated i.p. with MMH dissolved in normal saline at pH 2.0. The dosage was 10 mg/kg b.w. Lung tissues were removed at the times indicated in Table 5 and assayed for DNA replication and repair in short-term organ cultures. The tissues were minced in cold, buffered saline and then incubated with ^{3}H-thymidine, hydroxyurea and 4NQO to damage cellular DNA and stimulate DNA repair.

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* Values for bladder are the total number of labelled cells in the epithelial layer of a 6 μm section. Each value is based on the average of counts of 2 serial sections of bladders from different animals. The sections were chosen from full cross-sections of the bladder.

TABLE 5.

DNA replication and repair in lung tissue of MMH-treated golden hamster. Values are ^{3}H-DPM per μgm of DNA, and are based on duplicate or triplicate assays.

	Time After Treatment with MMH			
	30 Min	24 Hr	72 Hr	96 Hr
Experiment #1				
Repair Control		207		172
Repair MMH		139		176
Replication Control		653		946
Replication MMH		875		988
Experiment #2				
Repair Control	208	221		
Repair MMH	244	109		
Replication Control	1340	829		
Replication MMH	1392	480		
Experiment #3				
Repair Control	157	501	188	
Repair MMH	265	476	132	
Replication Control	860	610	1106	
Replication MMH	1002	1401	1404	

Measurement of ^{3}H-thymidine incorporation under these conditions is taken as an indication of DNA repair activity, and incorporation of ^{3}H-thymidine in the absence of hydroxyurea and 4NQO is a measure of DNA replication in preparation for mitosis.

Experimental Results and Conclusions

The results of 3 experiments are shown in Table 5. They suggest that there may be some suppression of repair activity at 24 hr after treatment with MMH, but the variability of the assay was such that no definite conclusion can be made. However, it can be concluded that a drastic inhibition or stimulation of either DNA repair or replication did not occur in the MMH treated animals.

EFFECT OF MMH GIVEN SYSTEMICALLY ON AMINOPYRINE-N-DEMETHYLASE OF MOUSE LIVER

Methods

Male C57Bl/6J mice, 2 mo old, were injected i.p. with MMH in 0.9% NaCl, pH 2.0, at doses of 10-30 mg/kg b.w. as indicated in Table 6 footnote. Aminopyrine-N-demethylase in cell-free homogenates of liver was measured as described by Mazel (1971).

TABLE 6.

Effect of MMH on amino-N-demethylase in mouse liver homogenates*

Time Post-Treatment	Nmoles of HCHO Control	MMH-Treated
Experiment #1		
30 min	76.5	161.5
2 hr	170	187
24 hr	119	141
Experiment #2		
30 min	130	139
2 hr	215	254
24 hr	161	187

Experimental Results and Conclusions

Treatment with 10 mg/kg b.w. appeared to produce a transient increase in N-demethylase activity when the enzyme was assayed in liver tissue removed 30 min post-treatment (Table 6). At later times, no significant difference was seen between controls and treated, indicating that there was no induction or loss of activity with time after treatment.

The reason for the transient stimulation of N-demethylase is not clear. Although this was observed in 3 out of 4 trials, the extent of increase was not consistent, and the levels of activity observed were variable between experiments.

Increasing doses of MMH up to 30 mg/kg b.w. did not produce an increased stimulation of N-demethylase in the livers of animals assayed at 30 min post-treatment (Table 7). The effect of these higher doses at later times after treatment has not been examined.

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* C57Bl/6J male mice were injected i.p. with MMH dissolved in 0.9% NaCl, pH 2.0, at a dose of 10 mg/kg b.w. Control animals received pH 2.0 saline only. At 30 min, 2 hr, and 24 hr, treated and control animals were killed and livers homogenized in tris-buffered 0.25 M sucrose, pH 7.4. Enzymatic activity in the 10,000 g supernatant was measured by the production of HCHO from aminopyrine as described by Mazel (1971). The reaction mixture contained 0.1 M KPO_4, 0.03 M $MgCl_2$, 0.03 M semicarbazide, 0.1 M NADP, 0.005 M glucose-6-PO_4, 0.01 M aminopyrine, and 4.5 mg protein in a total volume of 4.5 ml. Protein was determined by the Lowry method. The values are nmoles of HCHO produced in 10 min incubation at 37° C, and are the average of duplicate or triplicate samples.

TABLE 7.

Effect of increasing doses of MMH on aminopyrine-N-demethylase in homogenates of mouse liver.*

Nmoles of HCHO			
Control	10 mg/kg	20 mg/kg	30 mg/kg
170	212	204	187

STUDIES WITH JP-5

Methods

Inhalation Exposure. Exposure of female Fisher 344 rats and C57Bl/6J mice was carried out at the THRU as part of the toxicity assessment protocol. The exposure was continuous at 2 concentrations (0.15 mg/liter and 0.75 mg/liter) over a period of 90 dy. At the conclusion of the exposure, 3 mice and 3 rats from each group along with 3 controls of each were shipped by air to UCI. The animals arrived in good condition.

^{3}H-Thymidine Labelling Index. In order to estimate cellular proliferation in various tissues, the animals were injected i.p. with ^{3}H-thymidine (^{3}H-TdR, 100 µCi/mouse, 200 µCi/rat). After 1 hr, selected tissues were removed, fixed in 10% buffered formaldehyde, sectioned and processed for autoradiography by conventional methods. Lungs were fixed in the inflated state by perfusion of the excised lung via the trachea with fixative. Paraffin sections were cut at 6 µm. Test slides were developed at 2 wk and showed very light labelling. Therefore, the remaining slides were held for 3 mo before development, and are presently being analyzed.

Tests for Induction of Liver and Lung Hydroxylases by JP-5. JP-5 was dissolved in peanut oil and administered i.p. to 2 mo old male C57Bl/6 mice at 100 mg/kg b.w. Beta-naphthaflavone (BNF), a known inducer of liver aryl hydrocarbon hydroxylase (AHH) was used as a positive control at a dose of 80 mg/kg. Control animals received peanut oil only.

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* C57Bl/6J male mice were injected with MMH in 0.9% NaCl, pH 2.0 at doses of 10, 20, or 30 mg/kg b.w. Controls received saline only. After 30 min, the livers were removed, and the N-demethylase activity in the homogenates was determined as described in the footnote to Table 6. The values are nmoles of HCHO produced in 10 min incubation at 37° C, and are the average of triplicate samples.

TABLE 8.

Aryl hydrocarbon hydroxylase (AHH) activity in liver and lung homogenates from C57Bl/6 mice treated with beta-naphthaflavone or JP-5. Homogenates were prepared 48 hr after treatment with 80 mg/kg b.w. BNF or 100 mg/kg b.w. JP-5, or the 2 substances given together. The values are nmoles of ^{3}H-benzo(a)pyrene metabolized in 1 min by 500 µgms of protein.

Experiment	Tissue	Control	BNF	JP-5	BNF & JP-5
1	Liver	2.3	16.0	3.6	24.5
		4.5	21.7	5.4	31.6
2	Liver	12.4	20.1	5.1	
		5.8	15.5	6.1	
3	Liver	22.5	20.3	9.3	
		11.3	52.1	18.7	
4	Lung	2.5	1.5	0.07	2.7
		1.5	1.5	0.58	1.4
		2.1	1.4	1.5	

AHH activity was measured in cell-free homogenates of liver and lung remove at 48 hr post-treatment. Homogenates were centrifuged at 10,000 g for 20 min to remove unbroken cells and mitochondria, and the supernatant was assayed for AHH activity. Enzyme activity was measured using ^{3}H-benzo(a)pyrene (^{3}H-BP) as substrate. The enzymatic removal of ^{3}H-BP and the appearance of ^3H_2O was determined according to Hayakawa (1973).

Test for the Induction by JP-5 of N-Demethylase in Mouse Liver. C57Bl/6 male mice were injected i.p. with JP-5 or BNF as above. Liver aminopyrine-N-demethylase was measured in cell-free tissue homogenates by the method described by Mazel (1971). Livers were removed from treated animals at 24 and 36 hr. Enzyme activity was assayed in the 10,000 g supernatant of the homogenates.

Experimental Results and Conclusions

^{3}H-Thymidine Labelling Index. Autoradiographs of tissue sections from animals exposed to JP-5 at the THRU are presently being analyzed. No data are yet available.

Test for Induction of AHH by JP-5. Treatment with BNF induced AHH activity in liver 2-5-fold, but treatment with JP-5 had no effect. The data, presented in Table 8, also show that the combined treatment of BNF plus JP-5 did not significantly affect the induction of AHH by BNF.

The animals showed no obvious ill-effects of the BNF or JP-5 treatments

Table 9.

Aminopyrine-N-demethylase activity in homogenates of mouse liver from animals treated with JP-5. C57Bl/6 mice were treated i.p. with JP-5 at 100 mg/kg b.w. or BNF at 80 mg/kg b.w. At 24 and 36 hr, homogenates of liver were prepared, and the aminopyrine-N-demethylase activity in the 10,000 g supernatant was assayed as described in the text. The values are nmoles of HCHO produced in 10 min at a protein concentration of 1 mg/ml.

Time of Assay	Control	BNF	JP-5
24 hr	204	289	348
	221	298	323
36 hr	85	306	481
	289	250	450

Test for Induction of Aminopyrine-N-demethylase by BNF and JP-5. A single experiment was done in which aminopyrine-N-demethylase was assayed in homogenates of mouse liver from animals treated 48 hr previously with BNF or JP-5 as described. The results (Table 9) indicate that this enzyme activity was not induced by BNF, but may have been slightly increased by the JP-5 treatment. This result suggests that JP-5 may contain pharmacologically-active substances. This experiment will be repeated in order to confirm or negate these preliminary findings.

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